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# Emerging *Helicobacter pylori* levofloxacin resistance and novel genetic mutation in Nepal

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## Abstract

**Background:** The prevalence of *Helicobacter pylori* antibiotic susceptibility in the Nepalese strains is untracked. We determined the antibiotic susceptibility for *H. pylori* and analyzed the presence of genetic mutations associated with antibiotic resistance in Nepalese strains.

**Results:** This study included 146 consecutive patients who underwent gastroduodenal endoscopy in Kathmandu, Nepal. Among 42 isolated *H. pylori*, there was no resistance to amoxicillin and tetracycline. In contrast, similar with typical South Asian patterns; metronidazole resistance rate in Nepalese strains were extremely high (88.1 %, 37/42). Clarithromycin resistance rate in Nepalese strains were modestly high (21.4 %, 9/42). Most of metronidazole resistant strains had highly distributed *rdxA* and *frxA* mutations, but were relative coincidence without a synergistic effect to increase the minimum inhibitory concentration (MIC). Among strains with the high MIC, 63.6 % (7/11) were associated with frameshift mutation at position 18 of *frxA* with or without *rdxA* involvement. However, based on next generation sequencing data we found that one strain with the highest MIC value had a novel mutation in the form of amino acid substituted at Ala-212, Gln-382, Ile-485 of *dppA* and Leu-145, Thr-168, Glu-117, Val-121, Arg-221 in *dapF* aside from missense mutations in full-length *rdxA*. Mutations at Asn-87 and/or Asp-91 of the *gyrA* were predominantly in levofloxacin-resistant strains. The *gyrB* mutation had steady relationship with the *gyrA* 87–91 mutations. Although three (44.4 %) and two (22.2 %) of clarithromycin resistant strains had point mutation on A2143G and A2146G, we confirmed the involvement of *rpl22* and *infB* in high MIC strains without an *23SrRNA* mutation.

**Conclusions:** The rates of resistance to clarithromycin, metronidazole and levofloxacin were high in Nepalese strains, indicating that these antibiotics-based triple therapies are not useful as first-line treatment in Nepal. Bismuth or non-bismuth-based quadruple regimens, furazolidone-based triple therapy or rifabutin-based triple therapy may become alternative strategy in Nepal.

**Keywords:** Nepal, Drug resistance, *Helicobacter pylori*, Genetic mutation

## Background

The achievement of *Helicobacter pylori* against very hostile environment colonized on the stomach of over half of the world's population enact as the most successful human pathogens coexisted nearly sixty thousands years [1]. Although most of individuals exhibit overt disease leading to the hypothesis that the bacterium might be harmless and commensally, chronic infection of *H. pylori* represents a key factor in the etiology of various gastrointestinal diseases including chronic gastritis, peptic ulcer and mucosa-associated lymphoid tissue lymphoma. The outcome of each individual infection is capricious, similar to the rate of progression of the gastric mucosal damage. However, further progression is halted by eradication [2]. A recent meta-analysis supported that *H. pylori* eradication adequately decreases the rate of gastric malignancy, and the magnitude of the protective impact is more

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noteworthy among individuals with higher baseline gastric cancer risk [3]. Nevertheless, the adequacy of the standard first-line regimen containing a proton pump inhibitor, amoxicillin (AMX) and clarithromycin (CAM) or metronidazole (MNZ) has been seriously challenged and eradication rates below 70 % have been accounted in numerous countries, including South Asia [4, 5].

*H. pylori* antibiotic resistance mechanisms have been recognized in view of the different site-specific mutations that can be distinguished by molecular methods. It is important as a premise for consideration of more rational antibiotic combinations. One mechanism of CAM resistance has been elucidated due to one of five well-known point mutations (A2142G, A2143G, A2142C, A2144T, T2717C and C2694A) in the *23SrRNA* [6, 7]. Our previous report demonstrated higher MICs associated with the synergic effect of mutated sequences in *infB* (*hp1048*), *rpl22* (*hp1314*) and A2143G [8]. Additionally, inactivation mutation including frameshift mutation, insertions and deletions of the *rdxA* (*hp0954*) and *frxA* (*hp0642*) [9]. Novel mutations including *rpsU* (*hp0562*) [10], *dppA* (*hp0298*), *dppB* (*hp0299*), *rps4* (*hp1294*), *ackA* (*hp0903*), *rnc* (*hp0662)* and *dapF* (*hp0566)* were associated with MNZ resistance [11]. On the other hand, the mechanism of fluoroquinolone resistance in *H. pylori* has been identified to be linked to mutations in the quinolone resistance determining regions of the *gyrA* and *gyrB*, coding of the DNA gyrase [12]. Dual mutations in *gyrA* is accounted for a greater impact, while *gyrB* frequently occurred alongside *gyrA* mutations [13].

Nepal is a small landlocked country in South Asia with a low incidence of gastric cancer (5.3 cases per 100,000 populations per year; GLOBOCAN 2012; http://globocan.iarc.fr). Although it was varied between studies (16.3–70.5 %) [14–19], we confirmed the prevalence of *H. pylori* infection is 38.4 % (56/146) using several diagnostic test that significantly related to source of drinking water [20]. The majority of strains are so-called Western-type-*cagA* in Nepal as similar to typical South Asian patterns [20]. However, the mountainous people of northern Kathmandu are culturally linked to the Buddhists of Tibet, have higher prevalence of *H. pylori* infection and high-risk gastric mucosal atrophy than those Kathmandu people, the capital and the largest urban agglomerate of Nepal [21]. It is suggested lay stress on the need for *H. pylori* eradication in Nepal. Local antibiotic resistances screening are a key to counter primary *H. pylori* treatment failure, thus, reduce possibility spreading of secondary antibiotic resistance [4].

The prevalence of *H. pylori* antibiotic susceptibility in the Nepalese strains is untracked. Table 1 summarized *H. pylori* antibiotics resistance rates in South Asia. Generally, South Asian countries are the high CAM and MNZ resistance prevalence region [5]. Moreover, India and Bangladesh

strains demonstrated emerging levofloxacin (LVX) resistance [22, 23], the second-line regimen drug and as a rescue treatment for *H. pylori* eradication. In recent years, antibiotic resistance is expanding overall [24, 25], it is critical to look at current drug resistance rates in Nepal. In this study, we aimed to determine the antibiotic susceptibility of *H. pylori* to CAM, MNZ, AMX, tetracycline (TCN), and LVX. Furthermore, we also determined the presence of genetic mutations associated antibiotic resistance in Nepalese strains.

## Methods

### Patients and H. pylori

This study included 146 consecutive patients (76 women and 70 men; mean age of 42.2 ± 15.7 years) consecutively from July 2012 to September 2012. The survey was conducted at the endoscopy services section of the Gastroenterology Department, Tribhuvan University Teaching Hospital (TUTH), Kathmandu, Nepal. Peptic ulcer diseases, including gastric and duodenal ulcers, were diagnosed by endoscopic observation, while chronic gastritis was determined by histologic examination. Exclusion criteria included a history of partial gastric resection, eradication therapy for *H. pylori*, and treatment with bismuth-containing compounds, H2-receptor blockers, or proton pump inhibitors (PPI) within four weeks before the study.

For *H. pylori* culture, antral biopsy specimens were homogenized and inoculated onto Mueller Hinton II Agar medium (Becton Dickinson, NJ, USA) supplemented with 7 % horse blood without antibiotics. The plates were incubated for up to 10 days at 37 °C under microaerophilic conditions (10 % $O_2$, 5 % $CO_2$, and 85 % $N_2$). *H. pylori* isolates were identified based on colony morphology; Gram staining results; and positive reactions for oxidase, catalase, and urease. Isolated strains were stored at –80 °C in Brucella Broth (Difco, NJ, USA) containing 10 % dimethyl sulfoxide and 10 % horse serum.

### Antibiotic susceptibility testing

E-test (Biomerieux, Marcy l'´Etoile, France) was used to determine the minimum inhibitory concentration (MIC) of AMX, MNZ, TCN, CAM, and LVX. Mueller-Hinton II Agar medium (Becton Dickinson) supplemented with 10 % defibrinated horse blood was used as culture media. The bacterial suspension, adjusted to be equivalent to a McFarland opacity standard of 3.0, was inoculated onto the plates. After 72 h of incubation, the MIC of each antibiotic was determined. Quality control was performed using *H. pylori* ATCC 43504. The resistance breakpoints were determined as described by the European Committee on Antimicrobial Susceptibility Testing (EUCAST; available in http://www.eucast.org/). Strains were considered to be

**Table 1** H. pylori antibiotics resistance rates in South Asia

| Ref | Country | City | Year | Patients | Methods | CAM | MNZ | LVX | TCN | AMX | Others |
|---|---|---|---|---|---|---|---|---|---|---|---|
| [22] | India | Gujarat | 2008–2011 | 80 | DDM | 58.8 % | 83.8 % | 72.5 % | 53.8 % | 72.5 % | Ciprofloxacin (50 %) |
| [33] | India | Multicentre | – | 259 | E-test | 44.7 % | 77.9 % | – | – | 32.8 % | – |
| [53] | India | Kolkata | 2000–2001 | 67 | ADM | 0.0 % | 85.1 % | – | 7.5 % | 0.0 % | Furazolidone (0.0 %) |
| [37] | India | North India | – | 68 | ADM | 11.8 % | 48.5 % | – | 16.2 % | 17.6 % | Furazolidone (22.1 %) |
| [34] | India | Varanasi | 2005–2006 | 63 | ADM | 4.7 % | 100.0 | | 0.0 % | 65.1 % | – |
| [32] | Pakistan | Karachi | 2005–2008 | 178 | NM | 36.0 % | 89.0 % | – | 12.0 % | 37.0 % | Ofloxacin (18.5 %) |
| [54] | Pakistan | Karachi | 2008–2013 | 92 | E-test | 5.4 % | 97.8 % | 16.2 % | 4.3 % | 2.2 % | Ofloxacin (30.1 %), Furazolidone (15.2 %) |
| [55] | Pakistan | Karachi | 2007–2009 | 92 | E-test | 32.6 % | 47.8 % | – | – | 2.2 % | – |
| [56] | Pakistan | Karachi | 2009–2010 | 162 | E-test | 37.0 % | – | – | – | – | Fluoroquinolone 62.3 % |
| [35] | Pakistan | Rawalpindi | 2011–2012 | 46 | E-test | 47.8 % | 73.9 % | – | 4.4 % | 54.3 % | Ciprofloxacin (13.0 %) |
| [57] | Bangladesh | Dhaka | 1999–2001 | 174 | ADM | 10.0 % | 77.5 % | – | 15.0 % | 6.6 % | – |
| [23] | Bangladesh | Dhaka | 2014 | 56 | ADM | 39.3 % | 94.6 % | 66.1 % | 0.0 % | 3.6 % | – |

*Abbreviations*: ADM Agar Dilution Method, DDM Disk diffusion method, E-test Epsilometer test, CAM clarithromycin, MNZ metronidazole, LVX levofloxacin, AMX amoxicillin, TCN tetracycline

resistant for MICs >0.125 mg/L for AMX, 0.25 mg/L for CAM, 8 mg/L for MNZ, and 1 mg/L for TCN and LVX.

### Molecular detection on resistant strains

Mutations in gyrA, gyrB, rdxA, frxA and 23S rRNA were assessed on antibiotic-resistant strains by polymerase chain reaction (PCR) based sequencing. H. pylori DNA was extracted from H. pylori cultured to confluence on MNZ-resistant strains, gyrA and gyrB for LVX-resistant strains and 23S rRNA peptidyl transferase for CAM-resistant strains were amplified using the primers on the Additional file 1: Table S1 as described previously [13, 26, 27]. As a control, we sequenced randomly selected 4-sensitive MNZ and LVX strains and 2-sensitive CAM strains. The PCR products were analyzed by gel electrophoresis using 1.5 % agarose gel containing ethidium bromide. The sequences were then generated to the published sequence of the H. pylori strain 26695 (GenBank accession number AE000511.1 GI: 6626253) using the MAFFT version 7 (available in http://mafft.cbrc.jp/alignment/server/) and confirmed by visual inspection.

To find other genetic mutations with high MIC values but not involving typical 23S rRNA, rdxA and frxA mutations, we also obtained full-length 23S rRNA, infB, rpl22 [8], rdxA, frxA, rpsU [10], dppA, dppB, rps4, ackA, rnc and dapF [11] from next-generation sequencing (NGS) data (MiSeq next-generation sequencer; Illumina, Inc., San Diego, CA). MiSeq output was integrated into contig sequences by CLC Genomics Workbench 7.0.4. Genomics Workbench was also used for gene prediction and translation to protein sequences.

### Statistical analysis

Discrete variables were tested using the chi-square test, while continuous variables were tested using the Mann–Whitney $U$ and $t$-tests. $P$ values < 0.05 were considered statistically significant. The SPSS statistical software package version 18.0 (SPSS, Inc., Chicago, IL) was used for all statistical analyses.

## Results

### Prevalence of antibiotic resistance

The prevalence of H. pylori infection was 37.7 % (55/146) based on histology confirmed by immunohistochemistry, whereas using culture it was 34.9 % (51/146) [20]. However, 9 isolates did not grow when subcultured onto Mueller Hinton II Agar medium from antibiotic selection plate. Finally, a total of 42 H. pylori strains were successfully isolated; consisting 16 male (age range, 17 to 77 years; mean age, 42.3 ± 18.9 years) and 26 female patients (age range, 17 to 69 years; mean age 43.3 ± 14.8 years). The patients consisted of 35 with chronic gastritis, 4 with peptic ulcer diseases and 3 with gastric cancer. Overall, only three strains showed sensitive to all antibiotics (7.14 %). Interestingly, there was no AMX- and TCN-resistant strains and these strains had low MIC predominant (90.5 % for 0.016 mg/L or less for AMX and for 0.25 mg/L or less for TCN, respectively) (Table 2). In contrast, similar with typical South Asian pattern [5]; MNZ resistance rate in Nepalese strains showed an emerging antimicrobial resistance pattern (88.1 %, 37/42) with MIC values 64 mg/L or more (26/37, 70.3 %, Fig. 1). In addition, although CAM resistance rate in Nepalese strains were modestly high (21.4 %, 9/42), we detected a high prevalence of LVX resistance (42.9 %, 18/42) with a high distribution of great MIC

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**Table 2** The distribution of antibiotic resistance of *H. pylori* Nepalese isolated strains by sex and age

| Antibiotic | All patients | Sex | | Age (years) | | | | |
|---|---|---|---|---|---|---|---|---|
| | | Female | Male | <29 | 30–39 | 40–49 | 50–59 | >60 |
| | (n = 42) | (n = 26) | (n = 16) | (n = 10) | (n = 7) | (n = 11) | (n = 7) | (n = 7) |
| AMX | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| CAM | 9 (21.4) | 7 (26.9) | 2 (12.5) | 2 (20.0) | 2 (28.6) | 2 (18.2) | 1 (14.3) | 2 (28.6) |
| MNZ | 37 (88.1) | 24 (92.3) | 13 (81.3) | 8 (80.0) | 6 (85.7) | 9 (81.8) | 7 (100.0) | 7 (100.0) |
| TNC | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) | 0 (0.0) |
| LVX | 18 (42.9) | 10 (38.5) | 8 (50.0) | 6 (60.0) | 2 (28.6) | 3 (27.3) | 2 (28.6) | 5 (71.4) |

*Abbreviations*: *AMX* amoxicillin, *CAM* clarithromycin, *MNZ* metronidazole, *TCN* tetracycline, *LVX* levofloxacin

values predominant (94.4 % of resistant strains showed 32 mg/L or more). Antibiotic resistance rate did not differ among different age groups, gender and clinical outcomes ($P$ >0.05).

Overall, there was no strain resistant to all tested antibiotics. Only five strains were resistant to triple antibiotics; CAM, MNZ, and LVX (Table 3). Among all strains, 28.6 % (12/42) showed dual-drug resistance to MNZ and LVX. Additionally, three strains (7.1 %) were resistant to CAM and MNZ. No differences were observed in clinical outcomes between single-drug and multidrug resistant infections ($P$ >0.05).

### Detection of *H. pylori* genes mutations associated with antimicrobial resistance

The two and three MNZ-resistant strains did not show PCR identifiable specific bands target of *rdxA* and *frxA*, respectively. Therefore, a total 35 *rdxA* and 34 *frxA* of

MNZ-resistant strains were analyzed in this study compared to 4-sensitive strains. Both of DNA sequence analysis of *rdxA* and *frxA* from MNZ-sensitive strains revealed intact reading frames (lacking nonsense mutations). Pairwise alignment identified that the MNZ-sensitive strains shared 94.5–97.3 % and 96.5–98.6 % identity with the reference strain, 26695 for *rdxA* and *frxA*, respectively. In contrast, most of the *rdxA* of MNZ-resistant strains contained missense mutations (12/37, 32.4 %) and nonsense mutation resulted premature stop codon (12/37, 32.4 %, Table 4). Moreover, *rdxA* alleles of 7 strains (18.9 %) contained nucleotide deletion and/or insertion that resulted in translational frameshift. The similar pattern with *rdxA* showed in *frxA* of MNZ-resistant that also contained missense mutations, premature stop codon and translational frameshift (11/37, 29.7 %; 4/37, 10.8 % and 17/37, 45.9 %, respectively). The association between these two genes was relative coincidence without a synergistic effect to increase MIC values.
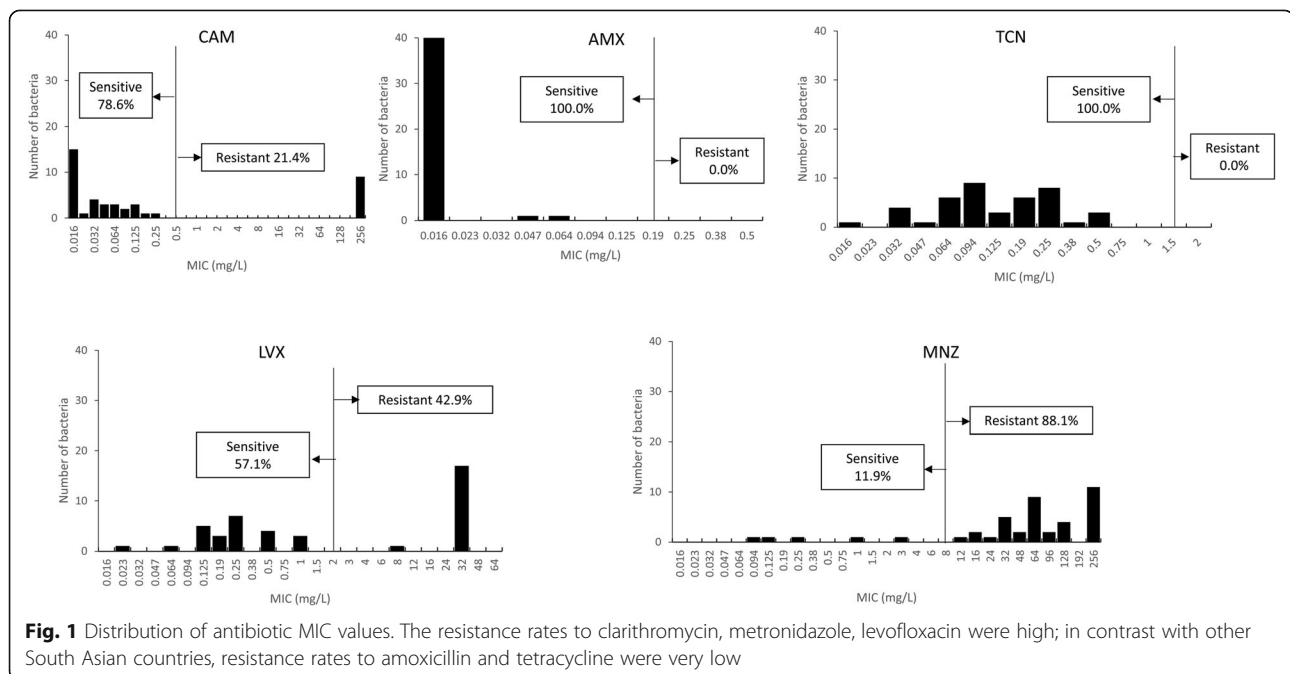


**Fig. 1** Distribution of antibiotic MIC values. The resistance rates to clarithromycin, metronidazole, levofloxacin were high; in contrast with other South Asian countries, resistance rates to amoxicillin and tetracycline were very low

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**Table 3** The antibiotic resistance patterns of *H. pylori* Nepalese strains

| Resistance pattern | N |
|---|---|
| Double drugs | |
| MNZ + LVX | 12 (28.6) |
| MNZ + CAM | 3 (7.1) |
| Triple drugs | |
| CAM + MNZ + LVX | 5 (11.9) |

*Abbreviations*: *CAM* clarithromycin, *MNZ* metronidazole, *LVX* levofloxacin

Among 7 strains with high MIC values (>256 mg/L or more), 63.6 % strains were associated with frameshift mutation at position 18 of *frxA* (7/11) with or without *rdxA* involvement. Interestingly, there was no mutation on any *rdxA* and *frxA* in one strain with high MIC values (Nepal120).

Based on the previous report [10, 11], we performed NGS of the Nepal120 strain (average sequencing depth was 249.8× and overall %GC was 39.0). Nonetheless, we could not obtain *ackA* and *rnc* from NGS data. Using strain 26695 and the control MNZ-sensitive strain Nepal145, we could not identify any mutations in full-length *frxA*, *dppB*, *rpsU* and *rps4*. In contrast, we revealed missense mutations in the full-length of *rdxA* at Arg-90, His-97, Pro-106 and Val-111. Moreover, we also confirmed involvement of novel mutated sequences in the form of amino acid substituted at Ala-212, Gln-382, Ile-485 of *dppA* and Leu-145, Thr-168, Glu-117, Val-121, Arg-221 in *dapF*.

There was no mutation on both of *gyrA* and *gyrB* sub-units among the control four LVX-sensitive strains. Among 18 LVX-resistant strains, 17 had amino acid variants at *gyrA* subunit (Table 5). The major well-known point mutations in the 91- and 87-positions were predominant (15/18, 83.3 %), including 9 of LVX-resistant strains (50.0 %) substituted amino acid at Asp-91, while six strains had amino acid substitution at Asn-87 (33.3 %). Other mutations included substituted amino acid at Ala-88, Ser-63 and Arg-130. On the other hand, only one strain exhibited amino acid substitution at Glu-483 in *gyrB* subunits. However, it is coincidence with *gyrB* without influence to increase of MIC values. There was no correlation between degree of LVX-resistance with the type and number of mutations in both genes.

Based on *23S rRNA* sequenced in the 9 CAM-resistant strains exhibited 3 (44.4 %) and 2 (22.2 %) had point mutation specifically on A2143G and A2146G, respectively. In contrast, we identified minimal nucleotide variation on the CAM-sensitive strains. Interestingly, there was no *23S rRNA* mutation in four strains with high MIC values (>256 mg/L or more). Based on the previous report [8], we also performed next generation sequencing of the Nepal90, Nepal110, Nepal114 and Nepal145

strains (average sequencing depth was 139.5×, 117.3×, 127.5×, 139.4×, respectively and overall %GC was 39.2, 39.0, 38.8, 38.9, respectively). Using strain 26695 and the control CAM-sensitive strain Nepal44, we could not identify any mutations in full-length *23S rRNA*. We confirmed the involvement of novel mutated sequences in C113T and G20A of *rpl22* and some interest mutations of *infB* such as G793A, C2669T, G2043T and C2784A (Table 6).

## Discussion

The AMX resistance rates in South Asia is diverse (Table 1), we revealed there was no AMX resistance from Nepalese isolates. Together with CAM or MNZ, AMX is the first-line regimen for treatment of *H. pylori* infection particularly as a secondary antibiotic in the low efficacy of CAM-based triple treatment zone [28–31]. Although in general the AMX resistance is rare, the increasing AMX primary resistance rates have been reported in the neighbor's country; India and Pakistan [22, 32–35]. AMX is one of the most commonly used antibiotics in recent years in Nepal as similar as ceftriaxone and gentamycin [36]. Additionally we observed no resistance to TCN, in contrast to studies from India and Pakistan [22, 32, 35, 37]. TCN is used as a salvage quadruple therapy [28, 38] and may be a useful alternative first-line regimen in Nepal. A strict regulation for anti-microbial use is necessary to counteract failure of these two essential antibiotics in Nepal.

Importantly, we observed a high prevalence of CAM resistance (21.4 %) in Nepalese strains. It is overabundance of the breaking points required by the Maastricht guidelines on *H. pylori* infection management (>15–20 %) [38, 39], consequently, CAM-based regimen may insufficient as a first line treatment for *H. pylori* eradication in Nepal. A meta-analysis demonstrated that utilization of triple therapy that consist of PPI, AMX, and CAM in cases of CAM resistance diminished the treatment efficacy by 66 % [40]. CAM is not a drug of choice in Nepalese physicians related a high cost [41]. Nonetheless, other macrolides consumption such as erythromycin and azithromycin used for lower respiratory infection in Nepal [41] and become essential risk for cross-resistance to CAM [42]. Additionally, similar with other countries in Asia, there was emerging resistance to MNZ in Nepal. MNZ is a simple medication often utilized to treat different diseases, for example, intestinal parasites and periodontal and gynecologic [43, 44]. In Asia, only Japan, Thailand, and Malaysia have populations with <40 % MNZ resistance [5]. Therefore, regimens including MNZ are not suitable and should not be chosen as first-line treatment in Nepal.

The T2183C and A2223G transformations have been frequently found to be the reason of observed CAM

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**Table 4** MIC of metronidazole resistant strains and the mutation of *rdxA* and *frxA* genes

| No | Strains | MIC (mg/L) | rdxA | frxA |
|---|---|---|---|---|
| 1 | 2 | 48 | 13frameshift | R86[a] |
| 2 | 4 | >256 | Q11[a] | 18frameshift |
| 3 | 5 | 64 | N73[a] | R3T, 54frameshift |
| 4 | 8 | 64 | Q11[a] | Q5[a] |
| 5 | 14 | 32 | R16H, L62V, K190[a] | P2E, R3P, M66I, A70V |
| 6 | 15 | 64 | K2N, 4frameshift | I44T, 47frameshift |
| 7 | 16 | 48 | E107R, 109frameshift | W137[a] |
| 8 | 18 | 128 | C148Y | undetermined |
| 9 | 29 | >256 | R16H, A80T, S108A | 18frameshift |
| 10 | 34 | 64 | undetermined | 106frameshift |
| 11 | 41 | 16 | R16H, R41K, 43frameshift | G76R, A152V |
| 12 | 49 | >256 | C140Y | 18frameshift |
| 13 | 52 | >256 | G189S | undetermined |
| 14 | 55 | 24 | L62V, S108A, S196N, Q197[a] | P41L, E176K |
| 15 | 61 | 12 | None | A15V, I144V, M66I |
| 16 | 64 | 32 | R16H, S108A, R176C, S196N | None |
| 17 | 70 | 32 | K60[a] | D2E, A85V, K178N |
| 18 | 74 | 64 | S45G | 6frameshift |
| 19 | 83 | >256 | M21V, A80T, Q119[a] | A70V |
| 20 | 84 | 16 | Q50[a] | R58H |
| 21 | 86 | 96 | Q50[a] | R25T, M66I, A154T |
| 22 | 89 | 32 | Q65[a] | A115V |
| 23 | 90 | 64 | 45frameshift | 18frameshift |
| 24 | 92 | >256 | C140Y | 18frameshift |
| 25 | 94 | 64 | Q50[a] | undetermined |
| 26 | 108 | 128 | R16L | 18frameshift |
| 27 | 110 | 32 | None | P41L |
| 28 | 113 | 128 | A40T | A16T, I44V, 70frameshift |
| 29 | 114 | >256 | Q16[a] | 18frameshift |
| 30 | 116 | >256 | G163D | 18frameshift |
| 31 | 120[b] | >256 | None | None |
| 32 | 123 | >256 | Q50[a] | V6[a] |
| 33 | 124 | >256 | D23G | 18frameshift |
| 34 | 137 | 128 | M56I, 201frameshift | 70frameshift |
| 35 | 140 | 96 | 60frameshift | 71frameshift |
| 36 | 141 | 64 | S43L | 72frameshift |
| 37 | 142 | 64 | undetermined | A15V |

Q11[a] means premature stop codon at Gln11; 13frameshift means frameshift mutation in the amino acid 13; R16H means amino acid substituted at Arg-16; None means no specific mutation; Undetermined is the strains that failed to show identifiable specific bands of *rdxA* or *frxA* target in PCR
[b]High MIC values strain without specific mutation in *rdxA* and *frxA* but contained mutation in *dppA* and *dapF*

resistance in Asian countries than those in Europe and North America [45]. However, in Nepal we observed the contribution of interest point change on A2143G and A2146G, as previous reports [46, 47]. The A2143G mutation has a much stronger effect than the A2142G and A2142C mutations [46]. Interestingly among several strains with high MIC values (>256 mg/L or more) without *23S rRNA* involvement, we confirmed novel mutated

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**Table 5** MIC of levofloxacin resistant strains and the mutation of *gyrA* and *gyrB* genes

| No | Strains | MIC (mg/L) | *gyrA* | *gyrB* |
|----|---------|------------|--------|--------|
| 1 | 2 | >32 | N87K | None |
| 2 | 5 | >32 | D91G | None |
| 3 | 8 | >32 | D91N | None |
| 4 | 16 | >32 | S63P, D91N | None |
| 5 | 18 | >32 | D91N | None |
| 6 | 29 | >32 | S63P, N87K, P188S | None |
| 7 | 38 | >32 | D99V | None |
| 8 | 49 | >32 | N87K, D91N, V172I | None |
| 9 | 55 | >32 | N87I | E483K |
| 10 | 70 | >32 | None | None |
| 11 | 86 | >32 | N87K | None |
| 12 | 89 | >32 | D91N, R130K | None |
| 13 | 90 | >32 | N87K | None |
| 14 | 120 | 8 | A88P | None |
| 15 | 123 | >32 | D91Y | None |
| 16 | 140 | >32 | D91N | None |
| 17 | 141 | >32 | D91N | None |
| 18 | 142 | >32 | S63P, R130K | None |

N87K means amino acid substituted at Asn-87; None means no specific mutation

sequences in *rpl22* and *infB* in the different position than previous publication [8]. Suggesting that *rpl22* and *infB* mutations might not only result in synergistic effects, but also could be independent causes of CAM resistance. On the other hand, we recognized diverse mutations involving the *rdxA* and *frxA* in the large part MNZ-resistant-strains; appear differently in relation to against MNZ-sensitive strains. Additionally, several strains with high MIC values were associated with a framing error in position 18 of *frxA* that may become a particular mutation site of Nepalese MNZ-resistant strains. Finally, we introduced the novel

mutation in *dppA* and *dapF* in addition to *rdxA* mutations but irrespective of *frxA* and *rpsU* mutations. Unlike *dapF* which is associated with biosynthesis of lysine and peptidoglycan [48], *dppA* has a role in the transportation of dipeptide ATP-binding cassette on a drug efflux pump [11] that eventually lead to MNZ resistance.

Several guidelines proposed that LVX ought to be utilized as a part of rescue treatment based on antibiotic susceptibility testing [28, 38, 49]. However, our findings showed a high prevalence of primary resistance to LVX that may also prompt cross-resistance with other fluoroquinolones. It is become a serious challenge and may reduce the efficacy of treatment with LVX-based regimens in Nepal. In addition, together with MNZ, LVX is the most commonly observed as multidrug resistance in Nepal. Furthermore, 5 strains were identified resistance to triple antibiotics. *H. pylori* strains harboring triple or quadruple resistance can hinder the choice and achievement of eradication regimens.

As similar with previous reports [50–52], point mutations at amino acid 87 (Asn to Lys, Tyr, or Ile) and 91 (Asp to Asn, Gly, or Tyr) were also mainly found for Nepalese strains. Interestingly, different transformations including substituted amino acid at Ser-63 and Arg-130 also associated with high MIC values. A few mutations and the coincidence of Glu-483 substitution in *gyrB* subunits with *gyrA* suggested a minimum influence of the *gyrB* mutations in Nepalese LVX-resistant strains. Finally, mutation analysis at position 18 of *frxA*, Asn-87 and/or Asp-91 of *gyrA*, A2143G and A2146G of *23SrRNA* will be useful as guiding follow-up of eradication after first-line regimens failure in Nepal. Recently, it was created a high accuracy DNA strip genotyping test combining PCR and hybridization that allows the molecular identification of mutations in the *gyrA* and *23SrRNA* within 6 h [47].

The number of samples in this study was relatively low, which certainly suggests the limitations of this study.

**Table 6** MIC of clarithromycin resistant strains and the mutation of *23S rRNA* gene

| No | Strains | MIC (mg/L) | *23S rRNA* | *rpl22* | *infB* |
|----|---------|------------|------------|---------|--------|
| 1 | 5 | >256 | A2143G | | |
| 2 | 29 | >256 | A2143G | | |
| 3 | 49 | >256 | A2146G | | |
| 4 | 89 | >256 | A2143G | | |
| 5 | 90 | >256 | None | C113T | C193A, T449C, G793A, T870G, C1157T, C1988T, C2669T, A2781G, C2784A |
| 6 | 92 | >256 | A2146G | | – |
| 7 | 110 | >256 | None | None | C133G, G139A, C821T, A2551G, 547del, 571del |
| 8 | 114 | >256 | None | G20A | A298G, G448A, G568A, A1108G, G2403T, C2669T |
| 9 | 145 | >256 | None | G20A | G8A, A403G, G793A, C810A, C878T, T1171G, G2043T, C2784A, G793A, C812T |

A2143G means point mutation at 2143 position; None means no specific mutation

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**Table 7** Regions with reported resistance and potential rescue regimens for *H. pylori* eradication in Asia [5]

| Resistance type | Country | First- and second-line therapy | | | | | | | Rescue therapy | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | CAM-based triple therapy | MNZ-based triple therapy | BIS-based quadruple therapy | non-BIS quadruple `concomitant` therapy | furazolidone-based triple therapy | Sequential therapy | Hybrid therapy | LVX-based triple therapy | RIF-based triple therapy |
| Low resistance to four antibiotics | Taiwan, Thailand, Malaysia | √ | √ | √ | √ | √ | √ | √ | √ | √ |
| High CAM resistance (>20 %) | Japan | | √ | √ | √ | √ | √ | √ | √ | √ |
| High MNZ resistance (>40 %) | China-Hong Kong, Saudi Arabia, Singapore, Bhutan | √ | | √ | √ | √ | √ | √ | √ | √ |
| High CAM and MNZ resistance | Turkey, Bahrain, Vietnam | | | √ | √ | | | √ | √ | √ |
| High CAM and LVX resistance | South Korea | | √ | √ | √ | √ | √ | √ | | √ |
| High CAM, MNZ, and LVX resistance | China-Beijing and Southeast China, Bangladesh, Nepal | | | √ | √ | √ | | | | √ |
| High CAM, MNZ, and AMX resistance | Indonesia | | | √ | | √ | | √ | √ | √ |
| High CAM, MNZ, AMX, and LVX (CIP) resistance | Iran, India, Pakistan | | | √ | | | | | | √ |

*Abbreviations*: *CAM* clarithromycin, *MNZ* metronidazole, *LVX* levofloxacin, *AMX* amoxicillin, *CIP* ciprofloxacin, *TCN* tetracycline, *RIF* Rifabutin

In addition, we only determined the presence of well-known genetic mutations associated with antibiotic resistance. However, our results could as a susceptibility-guided treatment in Nepal. High prevalence of CAM, MNZ and LVX resistance in Nepal results in prerequisite for utilizing other alternative strategies, for example, bismuth or non-bismuth-based quadruple regimens or rifabutin-based triple therapy is fundamental in Nepal (Table 7) [5]. Additional clinical trials are required to enhance the rate of successful eradication in Nepal.

## Conclusions

We revealed the rates of resistance to CAM, MNZ, and LVX were high in Nepal, which recommends that CAM-, MNZ-, and LVX-based triple therapies are not useful as first-line treatment in Nepal. TCN can be still utilized, albeit local information regarding its successful eradication rate is inadequate. Bismuth or non-bismuth-based quadruple regimens, furazolidone-based triple therapy or rifabutin-based triple therapy may become alternative strategy after first-line regimens failure in Nepal.

## Additional file

**Additional file 1: Table S1.** The oligonucleotide primers for amplifying *rdxA, frxA, gyrA, gyrB* and *23S rRNA*. (DOCX 14 kb)

### Abbreviations
AMX: Amoxicillin; CAM: Clarithromycin; MIC: Minimum inhibitory concentration; MNZ: Metronidazole; PCR: Polymerase chain reaction; PPI: Proton pump inhibitors; TCN: Tetracycline; TUTH: Tribhuvan University Teaching Hospital

### Availability of data and materials
The detail data and materials available on request (yyamaoka@oita-u.ac.jp). Nucleotide sequence data reported are available under the DDBJ accession numbers LC184279-LC184300, LC184302-LC184422 and LC184494-LC184511.

### Authors' contributions
YY and PKS designed the study; YY, and MM performed data analysis, data interpretation, and wrote the manuscript. YY, RPS, PKS, PS, MM contributed to data acquisition. YY revised the manuscript to include important content. All authors read and approved the final version of the manuscript.

### Competing interests
Potential competing interests: The authors declare that they have no competing interests.

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### References
1. Linz B, Balloux F, Moodley Y, Manica A, Liu H, Roumagnac P, Falush D, Stamer C, Prugnolle F, van der Merwe SW, et al. An African origin for the intimate association between humans and *Helicobacter pylori*. Nature. 2007; 445:915–8.
2. Graham DY. *Helicobacter pylori* update: gastric cancer, reliable therapy, and possible benefits. Gastroenterology. 2015;148:719–31. e3.
3. Lee YC, Chiang TH, Chou CK, Tu YK, Liao WC, Wu MS, Graham DY. Association between *Helicobacter pylori* eradication and gastric cancer incidence: A systematic review and meta-analysis. Gastroenterology. 2016; 150:1113–24. e5.
4. Papastergiou V, Georgopoulos SD, Karatapanis S. Treatment of *Helicobacter pylori* infection: meeting the challenge of antimicrobial resistance. World J Gastroenterol. 2014;20:9898–911.
5. Miftahussurur M, Yamaoka Y. Appropriate first-line regimens to combat *Helicobacter pylori* antibiotic resistance: an Asian perspective. Molecules. 2015;20:6068–92.
6. Suzuki RB, Lopes RA, da Camara Lopes GA, Hung Ho T, Speranca MA. Low *Helicobacter pylori* primary resistance to clarithromycin in gastric biopsy specimens from dyspeptic patients of a city in the interior of Sao Paulo, Brazil. BMC Gastroenterol. 2013;13:164.
7. Stone GG, Shortridge D, Flamm RK, Versalovic J, Beyer J, Idler K, Zulawinski L, Tanaka SK. Identification of a *23S rRNA* gene mutation in clarithromycin-resistant *Helicobacter pylori*. Helicobacter. 1996;1:227–8.
8. Binh TT, Shiota S, Suzuki R, Matsuda M, Trang TT, Kwon DH, Iwatani S, Yamaoka Y. Discovery of novel mutations for clarithromycin resistance in *Helicobacter pylori* by using next-generation sequencing. J Antimicrob Chemother. 2014;69:1796–803.
9. Gerrits MM, van der Wouden EJ, Bax DA, van Zwet AA, van Vliet AH, de Jong A, Kusters JG, Thijs JC, Kuipers EJ. Role of the *rdxA* and *frxA* genes in oxygen-dependent metronidazole resistance of *Helicobacter pylori*. J Med Microbiol. 2004;53:1123–8.
10. Binh TT, Suzuki R, Trang TT, Kwon DH, Yamaoka Y: Search for Novel Candidate Mutations for Metronidazole Resistance in *Helicobacter pylori* Using Next-Generation Seq. Antimicrob Agents Chemother. 2015;59:2343–8.
11. Li AQ, Dai N, Yan J, Zhu YL. Screening for metronidazole-resistance associated gene fragments of *H. pylori* by suppression subtractive hybridization. World J Gastroenterol. 2007;13:1847–50.
12. Rimbara E, Noguchi N, Kawai T, Sasatsu M. Fluoroquinolone resistance in *Helicobacter pylori*: role of mutations at position 87 and 91 of GyrA on the level of resistance and identification of a resistance conferring mutation in GyrB. Helicobacter. 2012;17:36–42.
13. Wang LH, Cheng H, Hu FL, Li J. Distribution of *gyrA* mutations in fluoroquinolone-resistant *Helicobacter pylori* strains. World J Gastroenterol. 2010;16:2272–7.
14. Thapa R, Lakhey M, Yadav PK, Kandel P, Aryal CM, Subba K. Histopathological study of endoscopic biopsies. JNMA J Nepal Med Assoc. 2013;52:354–6.
15. Shrestha S, Paudel P, Pradhan GB, Shrestha L, Bhattachan CL. Prevalence study of *H. pylori* infection in dyspeptic patients coming to Nepal Medical College Teaching Hospital, Jorpati, Kathmandu. Nepal Med Coll J. 2012;14: 229–33.
16. Dhakhwa R, Acharya IL, Shrestha HG, Joshi DM, Lama S, Lakhey M. Histopathologic study of chronic antral gastritis. J Nepal Health Res Counc. 2012;10:57–60.
17. Matsuhisa T, Miki M, Yamada N, Sharma SK, Shrestha BM. *Helicobacter pylori* infection, glandular atrophy, intestinal metaplasia and topography of chronic active gastritis in the Nepalese and Japanese population: the age, gender and endoscopic diagnosis matched study. Kathmandu Univ Med J (KUMJ). 2007;5:295–301.
18. Makaju RK, Tamang MD, Sharma Y, Sharma N, Koju R, Ashraf M. Prevalence of *Helicobacter pylori* in Dhulikhel Hospital, Kathmandu University Teaching Hospital: a retrospective histopathologic study. Kathmandu Univ Med J (KUMJ). 2005;3:355–9.
19. Shrestha R, Koirala K, Raj KC, Batajoo KH. *Helicobacter pylori* infection among patients with upper gastrointestinal symptoms: prevalence and relation to endoscopy diagnosis and histopathology. J Family Med Prim Care. 2014;3:154–8.
20. Miftahussurur M, Sharma RP, Shrestha PK, Suzuki R, Uchida T, Yamaoka Y. Molecular Epidemiology of *Helicobacter pylori* Infection in Nepal: Specific Ancestor Root. PloS One. 2015;10:e0134216.
21. Miftahussurur M, Sharma RP, Shrestha PK, Maharjan RK, Shiota S, Uchida T, Sato H, Yamaoka Y. *Helicobacter pylori* infection and gastric mucosal atrophy in two ethnic groups in Nepal. Asian Pac J Cancer Prev. 2015;16:7911–6.
22. Pandya HB, Agravat HH, Patel JS, Sodagar N. Emerging antimicrobial resistance pattern of *Helicobacter pylori* in central Gujarat. Indian J Med Microbiol. 2014;32:408–13.
23. Aftab H, Miftahussurur M, Subsomwong P, Ahmed F, Khan AA, Yamaoka Y. *Helicobacter pylori* antibiotic susceptibility patterns in Bangladesh: Emerging levofloxacin resistance. J Infect Dev Ctries. 2016;10:245–53.
24. Kobayashi I, Murakami K, Kato M, Kato S, Azuma T, Takahashi S, Uemura N, Katsuyama T, Fukuda Y, Haruma K, et al. Changing antimicrobial susceptibility epidemiology of *Helicobacter pylori* strains in Japan between 2002 and 2005. J Clin Microbiol. 2007;45:4006–10.
25. Cuadrado-Lavin A, Salcines-Caviedes JR, Carrascosa MF, Mellado P, Monteagudo I, Llorca J, Cobo M, Campos MR, Ayestaran B, Fernandez-Pousa A, et al. Antimicrobial susceptibility of *Helicobacter pylori* to six antibiotics currently used in Spain. J Antimicrob Chemother. 2012;67:170–3.
26. Kwon DH, Pena JA, Osato MS, Fox JG, Graham DY, Versalovic J. Frameshift mutations in *rdxA* and metronidazole resistance in North American *Helicobacter pylori* isolates. J Antimicrob Chemother. 2000;46:793–6.
27. Ho SL, Tan EL, Sam CK, Goh KL. Clarithromycin resistance and point mutations in the *23S rRNA* gene in *Helicobacter pylori* isolates from Malaysia. J Dig Dis. 2010;11:101–5.
28. Fock KM, Katelaris P, Sugano K, Ang TL, Hunt R, Talley NJ, Lam SK, Xiao SD, Tan HJ, Wu CY, et al. Second Asia-Pacific consensus guidelines for *Helicobacter pylori* infection. J Gastroenterol Hepatol. 2009;24:1587–600.
29. Kim SG, Jung HK, Lee HL, Jang JY, Lee H, Kim CG, Shin WG, Shin ES, Lee YC, Korean College of H, et al. [Guidelines for the diagnosis and treatment of *Helicobacter pylori* infection in Korea, 2013 revised edition]. Korean J Gastroenterol. 2013;62:3–26.
30. Asaka M. A new approach for elimination of gastric cancer deaths in Japan. Int J Cancer. 2013;132:1272–6.
31. Chinese Society of Gastroenterology CSGoHp, Liu WZ, Xie Y, Cheng H, Lu NH, Hu FL, Zhang WD, Zhou LY, Chen Y, Zeng ZR, et al. Fourth Chinese National Consensus Report on the management of *Helicobacter pylori* infection. J Dig Dis. 2013;14:211–21.
32. Khan A, Farooqui A, Manzoor H, Akhtar SS, Quraishy MS, Kazmi SU. Antibiotic resistance and *cagA* gene correlation: a looming crisis of *Helicobacter pylori*. World J Gastroenterol. 2012;18:2245–52.
33. Thyagarajan SP, Ray P, Das BK, Ayyagari A, Khan AA, Dharmalingam S, Rao UA, Rajasambandam P, Ramathilagam B, Bhasin D, et al. Geographical difference in antimicrobial resistance pattern of *Helicobacter pylori* clinical isolates from Indian patients: Multicentric study. J Gastroenterol Hepatol. 2003;18:1373–8.
34. Singh V, Mishra S, Maurya P, Rao G, Jain AK, Dixit VK, Gulati AK, Nath G. Drug resistance pattern and clonality in *H. pylori* strains. J Infect Dev Ctries. 2009;3:130–6.
35. Rasheed F, Campbell BJ, Alfizah H, Varro A, Zahra R, Yamaoka Y, Pritchard DM. Analysis of clinical isolates of *Helicobacter pylori* in Pakistan reveals high

degrees of pathogenicity and high frequencies of antibiotic resistance. Helicobacter. 2014;19:387–99.

36. Basnyat B, Pokharel P, Dixit S, Giri S. Antibiotic Use, Its Resistance in Nepal and Recommendations for Action: A Situation Analysis. J Nepal Health Res Counc. 2015;13:102–11.

37. Gehlot V, Mahant S, Mukhopadhyay AK, Das K, De R, Kar P, Das R. Antimicrobial susceptibility profiles of *Helicobacter pylori* isolated from patients in North India. J Global Antimicrob Resist. 2016;5:51–6.

38. Malfertheiner P, Megraud F, O'Morain C, Bazzoli F, El-Omar E, Graham D, Hunt R, Rokkas T, Vakil N, Kuipers EJ. Current concepts in the management of *Helicobacter pylori* infection: the Maastricht III Consensus Report. Gut. 2007;56:772–81.

39. Malfertheiner P, Megraud F, O'Morain CA, Atherton J, Axon AT, Bazzoli F, Gensini GF, Gisbert JP, Graham DY, Rokkas T, et al. Management of *Helicobacter pylori* infection–the Maastricht IV/Florence Consensus Report. Gut. 2012;61:646–64.

40. Fischbach L, Evans EL. Meta-analysis: the effect of antibiotic resistance status on the efficacy of triple and quadruple first-line therapies for *Helicobacter pylori*. Aliment Pharmacol Ther. 2007;26:343–57.

41. Pradhan S, Jauhari AC. A study of antibiotics used in adult respiratory disorders in Kathmandu and Bhaktapur. Nepal Med Coll J. 2007;9:120–4.

42. Zuckerman JM. Macrolides and ketolides: azithromycin, clarithromycin, telithromycin. Infect Dis Clin North Am. 2004;18:621–49. xi-.

43. Megraud F. *H. pylori* antibiotic resistance: prevalence, importance, and advances in testing. Gut. 2004;53:1374–84.

44. Megraud F, Coenen S, Versporten A, Kist M, Lopez-Brea M, Hirschl AM, Andersen LP, Goossens H, Glupczynski Y, Study Group p. *Helicobacter pylori* resistance to antibiotics in Europe and its relationship to antibiotic consumption. Gut. 2013;62:34–42.

45. Ierardi E, Giorgio F, Losurdo G, Di Leo A, Principi M. How antibiotic resistances could change *Helicobacter pylori* treatment: A matter of geography? World J Gastroenterol. 2013;19:8168–80.

46. De Francesco V, Margiotta M, Zullo A, Hassan C, Troiani L, Burattini O, Stella F, Di Leo A, Russo F, Marangi S, et al. Clarithromycin-resistant genotypes and eradication of *Helicobacter pylori*. Ann Intern Med. 2006;144:94–100.

47. Cambau E, Allerheiligen V, Coulon C, Corbel C, Lascols C, Deforges L, Soussy CJ, Delchier JC, Megraud F. Evaluation of a new test, genotype HelicoDR, for molecular detection of antibiotic resistance in *Helicobacter pylori*. J Clin Microbiol. 2009;47:3600–7.

48. Richaud C, Higgins W, Mengin-Lecreulx D, Stragier P. Molecular cloning, characterization, and chromosomal localization of dapF, the Escherichia coli gene for diaminopimelate epimerase. J Bacteriol. 1987;169:1454–9.

49. Chey WD, Wong BC. Practice Parameters Committee of the American College of G: American College of Gastroenterology guideline on the management of *Helicobacter pylori* infection. Am J Gastroenterol. 2007;102:1808–25.

50. Nishizawa T, Suzuki H, Kurabayashi K, Masaoka T, Muraoka H, Mori M, Iwaski E, Kobayashi I, Hibi T. Gatifloxacin resistance and mutations in *gyrA* after unsuccessful *Helicobacter pylori* eradication in Japan. Antimicrob Agents Chemother. 2006;50:1538–40.

51. Garcia M, Raymond J, Garnier M, Cremniter J, Burucoa C. Distribution of spontaneous gyrA mutations in 97 fluoroquinolone-resistant *Helicobacter pylori* isolates collected in France. Antimicrob Agents Chemother. 2012;56:550–1.

52. Teh X, Khosravi Y, Lee WC, Leow AH, Loke MF, Vadivelu J, Goh KL. Functional and molecular surveillance of *Helicobacter pylori* antibiotic resistance in Kuala Lumpur. PloS One. 2014;9:e101481.